Phosphorylase Kinase from Rabbit Skeletal Muscle: Phosphorylation of κ-Casein[1]

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Phosphorylase kinase (EC 2.7.1.38) from rabbit skeletal muscle catalyzed the phosphorylation of κ-casein (M_r = 19,000) to a stoichiometry of approximately one mole of phosphate per mole of κ-casein. The reaction rate was modified by several factors known to influence phosphorylase kinase activity: (1) stimulation by Ca^{2+}, (2) further stimulation by added calmodulin in the presence of Ca^{2+}, (3) higher activity at pH 8.2 than at pH 6.8, and (4) activation, measured at pH 6.8, following partial proteolysis of the kinase by trypsin. The maximal rate of phosphorylation of κ-casein by nonactivated phosphorylase kinase in the presence of Ca^{2+} at pH 8.2 was 16 nmol/min/mg, and the κ-casein concentration for half-maximal activity was 80 μM. The phosphorylation of other components of whole casein, α_{s1}-casein and β-casein, by phosphorylase kinase was detectable but much slower than the reaction with κ-casein. When whole casein was used as a substrate, κ-casein was identified by electrophoresis as a major phosphorylated species. The amino acid residues in κ-casein modified by phosphorylase kinase were shown to be serines. The present work extends the known substrates of phosphorylase kinase to include a well-characterized protein that may prove an interesting model substrate. Furthermore, this report emphasizes that whole casein, because of its heterogeneity, is a poor substrate to use in characterizing the substrate specificities of protein kinases, since different "casein kinases" may be specific for different components of whole casein.

Phosphorylase kinase is considered to have a central role in regulating glycogen metabolism in a variety of tissues (1-3). The muscle enzyme, which has been studied extensively, is a complex protein composed of four subunit types, α, β, γ (4, 5), and δ (6), the last named being the same as the Ca^{2+}-binding regulator protein (calmodulin or CDR) that controls a number of cellular processes (7, 8). Reversible activation of muscle phosphorylase kinase by Ca^{2+} is an important property of the enzyme (9, 10); current hypotheses ascribe the mediation of this Ca^{2+} sensitivity to the calmodulin intrinsically associated with native phosphorylase kinase (11). Also, added calmodulin, in the presence of Ca^{2+}, causes a further stimulation of phosphorylase kinase by a process that can be distinguised from the effect of Ca^{2+} alone (11-13). Cohen (11, 12) has proposed that a second calmodulin molecule binds to phosphorylase kinase and is involved in this activation.

Initially, the only significant substrate for the enzyme was believed to be phosphorylase, although early work had indicated that the enzyme also phosphorylated whole casein (14). Subsequently, phosphorylase kinase was reported to phosphorylate troponin I (15) and troponin T (16). Recently, we showed that glycogen synthase was also a substrate for phos-

[1] Supported in part by Grants AM 27221 and AM 27240 from the Public Health Service, the Wayne Newton Research Grant from the American Diabetes Association, and the Grace M. Showalter Foundation.

[2] Recipient of a Fellowship (AM 06218) from the Public Health Service.

phorylase kinase (17, 18), a finding that has been confirmed in three other laboratories (19–21). Furthermore, the phosphorylation of a number of synthetic peptides by phosphorylase kinase has been studied (see Ref. (1) for a review). While investigating the substrate specificities of phosphorylase kinase and other glycogen synthase kinases (22), we confirmed the earlier report that casein can be phosphorylated by phosphorylase kinase. However, the use of whole casein in protein kinase research is inconclusive since whole casein is a heterogeneous mixture of proteins. The components of whole casein are α_{s1}-casein, β-casein, κ-casein, and α_{s2}-casein, which occur in the proportion of 38, 38, 13, and 11%, respectively. Therefore, we have examined individual fractions of casein as well as whole casein to determine which components could be phosphorylated. We report here that κ-casein is the best substrate for phosphorylase kinase and is a major phosphorylated species in whole casein phosphorylated by phosphorylase kinase. Since the amino acid sequence of κ-casein is known (23, 24), this work extends the list of well-defined protein substrates for phosphorylase kinase and should provide a useful model for the study of phosphorylase kinase action. In addition, the need for caution when whole casein is used in the determination of protein kinase specificity is discussed.

EXPERIMENTAL PROCEDURES

Protein purification. Phosphorylase kinase was purified from rabbit muscle following the method of Cohen (5), except that 0.5 mM phenylmethylsulfonyl fluoride and 0.05 mM *N*-α-*p*-tosyl-L-lysine chloromethylketone-HCl were present in all buffers. The resulting enzyme had properties similar to those described previously (see Refs. (18, 22)). Phosphorylase was purified from rabbit muscle by a slight modification of the method of Fischer and Krebs (25) and prepared for use as a kinase substrate as described previously (18, 22). Casein was isolated from the milk of individual cows homozygous for particular variants. Whole casein was precipitated from skim milk with 1.0 N HCl at pH 4.6 and room temperature. The mixture was filtered through a flannel bag and the precipitate was washed with distilled water (pH 4.6). The precipitate was dispersed in water by adding 1.0 N NaOH to a final pH of 7.5. The above procedure was repeated twice and the final solution was freeze dried. The whole casein used contained the variants α_{s1}-casein B, β-casein A^1, and κ-casein A. Individual casein components were purified according to published procedures: α_{s1}-casein (27, 28), β-casein (29), and κ-casein (30).

Assay of protein phosphorylation. The chromatographic method of Huang and Robinson (31) was used to measure protein phosphorylation (see Refs. (18, 22) for further details). Standard reaction mixtures (50 μl) contained final concentrations of 25 mM Tris, 20 mM β-glycerol-*P*, 0.5 mM EDTA, 0.22 mM EGTA[3], 0.2 mM [γ-^{32}P]ATP (700–1000 cpm/pmol), 6 mM $Mg(CH_3CO_2)_2$, 15 mM β-mercaptoethanol, 1 mg/ml protein substrate, phosphorylase kinase, and, when present, 0.5 mM $CaCl_2$. The reaction was carried out for 10 min at 30°C and at pH 8.2.

Polyacrylamide gel electrophoresis. Samples of phosphorylated caseins to be analyzed by electrophoresis were dialyzed against 10 mM Tris-Cl, 1 mM EDTA, and 0.4 mM EGTA, pH 7.8, to remove the bulk of the unreacted [γ-^{32}P]ATP. The sample was then diluted 1:2 in buffer containing 4 M urea, 19 mM Tris-Cl, pH 8.5, either with or without 1% (v/v) β-mercaptoethanol. The gels, 7.5% acrylamide, were prepared by mixing 10.1 ml of a solution containing 222 g/liter acrylamide, 6 g/liter *N*,*N*'-methylenebis(acrylamide); 10 ml of 1.13 M Tris-Cl, 45 μl of TEMED; 7.21 g of urea; when noted, 0.4% (v/v) β-mercaptoethanol was present. This solution was adusted to pH 9.5 with HCl and brought to 30 ml. Polymerization was initiated by the addition of 0.15 ml of 0.15 g/ml ammonium persulfate. The reservoir buffer was 0.1 M glycine brought to pH 9.5 with Tris base, and, where indicated, contained also 0.4% (v/v) β-mercaptoethanol. Tube gels, 0.6-cm diameter, were run at 2 mA/tube. Proteins were identified by staining with Coomassie blue following standard procedures (32). Gels to be analyzed for radioactivity were cut into 1-mm slices which were immersed in 1 ml of 5 g/liter 2,5-diphenyloxazole in toluene before counting in a liquid scintillation counter.

Identification of phosphorylated amino acids. κ-Casein (32 μg) was phosphorylated by phosphorylase kinase, as described above, at pH 8.2 in the presence of Ca^{2+}. The protein was precipitated with 10% (w/v) trichloroacetic acid and left 30 min at 0°C. The precipitate was washed with cold 10% (w/v) trichloroacetic acid and finally with cold ether, before being taken up in 0.3 ml 6 N HCl. The tube was sealed under vacuum and incubated at 110°C for 4 h. After drying under vacuum, the residue was dissolved in 10 μl of electrophoresis buffer. An aliquot, 4 μl, was spotted on a cellulose thin-layer plate (Eastman 13255) which

[3] Abbreviations used: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; TEMED, *N*,*N*,*N*',*N*'-tetramethylethylenediamine.

was then electrophoresed at 1000 V for 45 min with buffer, pH 3.5, composed of acetic acid:pyridine:water, 50:5:945 (33). Standards of phosphoserine, phosphothreonine, and phosphotyrosine were run, and their migration, toward the anode, determined by spraying the plate with ninhydrin. Autoradiograms were made by placing the thin-layer plate in contact with Cronex 4 X-ray film (DuPont).

Other materials and methods. Reagents for polyacrylamide gel electrophoresis were from Bio-Rad. [γ-^{32}P]ATP was from New England Nuclear. Calmodulin was kindly supplied by Dr. Kretsinger, University of Virginia. Concentrations of casein. ATP, and phosphorylase were determined from uv absorption. At 280 nm, $E^{1\%}_{1\,cm}$ is 1.02, 0.46, and 1.22 for α_{s1}-casein (30), β-casein (34), and κ-casein (30), respectively. The concentration of phosphorylase kinase was estimated by the method of Lowry *et al.* (35) using bovine serum albumin as standard. Phosphotyrosine was prepared as described by Rothberg *et al.* (36).

RESULTS

Specificity of casein phosphorylation by phosphorylase kinase. Purified caseins as well as whole casein and phosphorylase kinase were compared as substrates for phosphorylase kinase (Table I). Of the caseins tested, κ-casein was a significantly better substrate than β-casein (A or B variant), α_{s1}-casein, or whole casein. Kinase activity toward all substrates was stimulated by the presence of Ca^{2+}. Under the conditions of this determination, phosphorylase was phosphorylated at almost 30 times the rate of κ-casein in the presence of Ca^{2+} and 13 times the rate in the absence of Ca^{2+}.

TABLE I

SPECIFICITY OF CASEIN PHOSPHORYLATION BY PHOSPHORYLASE KINASE

Substrate	Phosphorylation rate (nmol/min/mg kinase)	
	$-Ca^{2+}$	$+Ca^{2+}$
κ-Casein	0.93	8.76
β-Casein B	0.10	0.67
β-Casein A	0.10	0.64
α_{s1}-Casein	0.14	0.64
Whole casein	0.24	0.61
Phosphorylase	12.6	255

Note. The phosphorylation of the indicated substrates, at 1 mg/ml, by phosphorylase kinase, in the presence and absence of Ca^{2+} at pH 8.2 was determined by the standard assay described under Experimental Procedures. Phosphorylase kinase was 50 μg/ml when β-casein B, β-casein A, α_{s1}-casein, or whole casein was substrate; 12.5 μg/ml when κ-casein was substrate; and 0.31 μg/ml when phosphorylase was substrate.

TABLE II

MODIFICATION OF PHOSPHORYLASE KINASE ACTIVITY TOWARD κ-CASEIN

Experimental conditions			Phosphorylation rate (nmol/min/mg kinase)		
pH	Ca^{2+}	Calmodulin	I	II	III
6.8	+	−		2.21	1.02
8.2	−	−	1.20	0.87	
8.2	+	−	7.49	7.65	
8.2	−	+	1.10		
8.2	+	+	13.72		
		After tryptic proteolysis			
6.8	+	−			4.65

Note. Phosphorylase kinase activity was measured as described under Experimental Procedures with conditions as indicated. When present, calmodulin was 26 μg/ml. Three separate experiments (I, II, and III) are combined in this table. Phosphorylase kinase was 25 μg/ml.

Effect of modifying phosphorylase kinase activity. Several known means for modifying the activity of phosphorylase kinase activity toward substrates, such as gycogen synthase and phosphorylase, were tested using κ-casein as substrate (Table II). As noted above, the reaction rate was stimulated by Ca^{2+}; the presence of 26 μg/ml calmodulin resulted in a further stimulation of nearly 100%. The reaction pH, an important determinant of phosphorylase kinase activity, was tested with κ-casein as the substrate. The activity was higher at pH 8.2 than at pH 6.8. Partial proteolysis by trypsin activates phosphorylase kinase, and this is especially evident when the activity is measured at pH 6.8. After incubation of phosphorylase kinase (2.5 mg/ml) with trypsin (0.5 μg/ml) for 30 min at 30°C, conditions that we have used previously to study the activation of phosphorylase kinase by this mechanism (18), a significant stimulation of the activity toward κ-casein was observed. From the experiments above, it is clear that con-

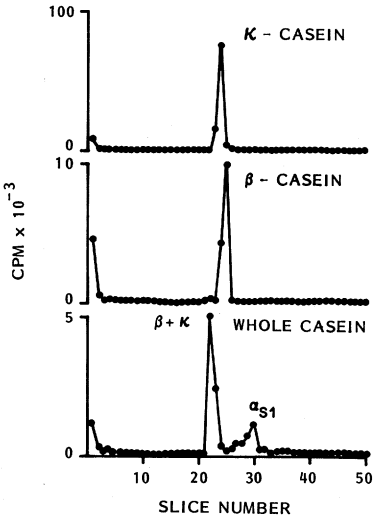

FIG. 1. Electrophoretic separation of phosphorylated caseins in the presence of β-mercaptoethanol. Whole casein, κ-casein, and β-casein B (1 mg/ml) were phosphorylated by phosphorylase kinase (50 μg/ml) as described under Experimental Procedures except that the time of incubation was increased to 150 min. Final stoichiometries of phosphorylation after incubation for 150 min were: whole casein, 0.035 (taking M_r = 20,000); κ-casein, 0.36; and β-casein B, 0.03. After dialysis to remove unreacted [γ-^{32}P]ATP, 50 μl of casein solution was added to 50 μl of 4 M urea, 10 mM Tris-Cl, pH 8.5, containing 1% (v/v) β-mercapthethanol. After 2 h at room temperature, a 40-μl sample was applied to gels containing 0.4% (v/v) β-mercaptoethanol and electrophoresed with reservoir buffer that also contained 0.4% (v/v) β-mercaptoethanol. Gels were analyzed after electrophoresis for the distribution of ^{32}P and parallel gels were stained for protein. Slice number 1 corresponds to the top of the gel. The migration of bromophenol blue corresponded to slice number 55.

ditions known to modify phosphorylase kinase activity toward its well-established substrates caused similar variations in the rate at which κ-casein was phosphorylated. We conclude, therefore, that phosphorylase kinase was indeed the enzyme responsible for the phosphorylation of κ-casein.

Verification of the phosphorylation of κ-casein. As for any novel protein phosphorylation, it was necessary to demonstrate unequivocally that κ-casein was the protein substrate which participated in the reaction. κ-Casein phosphorylated by phosphorylase kinase was examined by polyacrylamide gel electrophoresis in the presence of urea and at an alkaline pH. In the presence of β-mercaptoethanol (Fig. 1), a single peak of radioactivity was found, coincident with the Coomassie-staining material. When the electrophoretic analysis was carried out in the absence of β-mercaptoethanol, most of radioactivity did not enter the gel; minor bands of higher mobility were also seen (Fig. 2). The radioactivity corresponded to the principal protein staining material at the top of the gel and faint, multiple bands of slightly higher mobility, which were incompletely resolved by the slicing of the gel. κ-Casein forms intermolecular disulfide bridges (37) to yield a heterogeneous collection of high-molecular-weight aggregates. Disulfide bridge formation can be prevented by reducing agents, such as β-mercaptoethanol. Thus the behavior of κ-casein on electrophoresis differs depending on whether β-mercaptoethanol is present.

Similar analyses of β-casein phosphorylated by phosphorylase kinase showed no such dependence of the mobility of the phosphorylated species on the presence of β-mercaptoethanol (Figs. 1 and 2). A certain proportion of radioactivity was observed in the first slice of the gel of β-casein whether β-mercaptoethanol was present or not (Figs. 1 and 2). The explanation for this was not apparent. Nonetheless, exclusion of β-mercaptoethanol provided a means to distinguish clearly β-casein from κ-casein in this separation system. In Figs. 1 and 2, the greater amount of radioactivity associated with κ-casein compared with either β-casein or whole casein indicated again that κ-casein was the best substrate under identical incubation conditions (see Table I). From these results we conclude that κ-casein undergoes phosphorylation during incubation with phosphorylase kinase.

Phosphorylation of whole casein by phosphorylase kinase. Since whole casein is a commonly used kinase substrate, we wished to determine which of the individ-

ual components would be phosphorylated by phosphorylase kinase. Phosphorylation would of course depend on the specificity for the different caseins, the relative proportions of the different components, and the incubation period. Whole casein, incubated with [γ-^{32}P]ATP and phosphorylase kinase, was analyzed by the electrophoretic technique described above, and in parallel with the purified β- and κ-caseins. In the presence of β-mercaptoethanol, one major peak of radioactivity and a second smaller peak were observed (Fig. 1), the latter corresponding to the migration of α_{s1}-casein. The major peak had a mobility close to those of β-casein and κ-casein (see Fig. 1) which were separated by less than the resolution of the slicing technique. In order to resolve β-casein from κ-casein, an identical aliquot of phosphorylated whole casein was electrophoresed in the absence of β-mercaptoethanol (Fig. 2). A large fraction of the radioactivity now had very low mobility, characterizing it as aggregated κ-casein. We

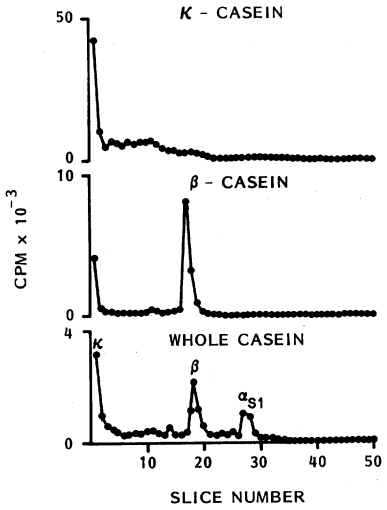

FIG. 2. Electrophoretic separation of phosphorylated caseins in the absence of β-mercaptoethanol. Aliquots of caseins from the same incubation described in the legend to Fig. 1 were analyzed by electrophoresis, identical to that described except that no β-mercaptoethanol was included. Slice number 1 corresponds to the top of the gel and the migration of bromophenol blue to slice number 54.

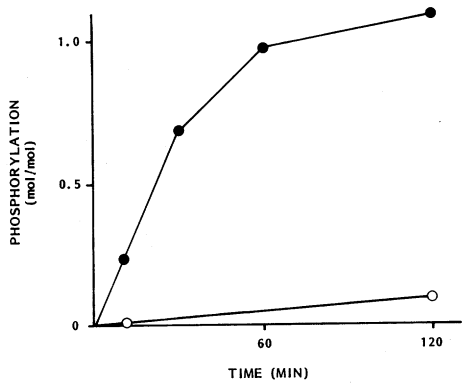

FIG. 3. Time course of κ-casein phosphorylation. The phosphorylation of κ-casein (1 mg/ml) by phosphorylase kinase (50 μg/ml) was followed at pH 8.2 in the presence of Ca^{2+} as described under Experimental Procedures (solid circles) (1 mg/ml). Values for a parallel incubation of whole casein are also shown (open circles).

conclude, then, that κ-casein was a major phosphorylated species when whole casein was phosphorylated by phosphorylase kinase.

Stoichiometry and kinetics of κ-casein phosphorylation. The phosphorylation of κ-casein (Fig. 3) proceeded to a stoichiometry of approximately one mole of phosphate per mole of κ-casein (taking M_r = 19,000; Ref. (38)). Figure 3 also shows that whole casein was phosphorylated at a much slower rate under the same conditions. The dependence of phosphorylase kinase activity on κ-casein concentration (Fig. 4) gave a maximal rate of 16 nmol/min/mg, with half-maximal activity corresponding to 80 μM substrate calculated from a Hill plot. The Hill slope was 1.2 but a more detailed kinetic study would be necessary to evaluate the significance of this deviation from hyperbolic kinetics.

Identification of amino acid residue phosphorylated in κ-casein. As described under Experimental Procedures κ-casein was phosphorylated by phosphorylase kinase using [γ-^{32}P]ATP, hydrolyzed, and subjected to thin-layer electrophoresis at pH 3.5 (33). As shown in Fig. 5, phosphoserine, but not phosphothreonine or phosphotyrosine, was identified in the hydrolysate.

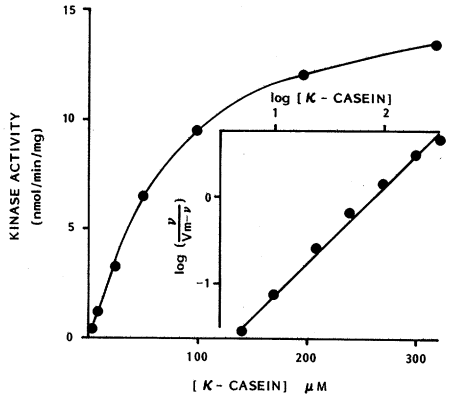

FIG. 4. Dependence of phosphorylase kinase activity on κ-casein concentration. Kinase activity was determined by standard methods, at pH 8.2, and in the presence of Ca^{2+}, with the indicated concentration of κ-casein. Phosphorylase kinase concentration was 25 μg/ml. The maximal rate was estimated from an Eadie–Hofstee plot. The abscissa of the Hill plot (see inset) is the logarithm of substrate concentration expressed as micromolarity. The straight line drawn in the Hill plot is the unweighted least-squares fit for the data taking the logarithm of κ-casein concentration as the independent variable.

DISCUSSION

The results establish that, of the individual components of whole casein, κ-casein is preferentially phosphorylated by phosphorylase kinase, thus defining a new, well-characterized protein substrate of phosphorylase kinase. As discussed in the introduction, the list of protein substrates for phosphorylase kinase is quite limited. The κ-casein concentration required for half-maximal activity, 80 μM, was quite low compared with corresponding values for other substrates (see Ref. (1)). The K_m for phosphorylase, for example, is of the order of 270 μM for nonactivated phosphorylase kinase (39). Insofar as such kinetic measures reflect binding, one would judge that the affinity of phosphorylase kinase or κ-casein is comparable to that for a physiological substrate like phosphorylase (one imagines that the phosphorylation of κ-casein by this kinase is not of physiological significance). In contrast, the maximal rate of κ-casein phosphorylation was low compared with phosphorylase, some 50 times lower in our hands. Clearly, κ-casein does not contain all the determinants of more effective substrates such as phosphorylase or glycogen synthase. Phosphorylation of κ-casein by phosphorylase kinase occurs at serine residues and this follows the pattern of the best-characterized sites of phosphorylase kinase action (1). The primary structure of κ-casein does not reveal any serines with a surrounding local sequence strikingly similar to those of either serine-14

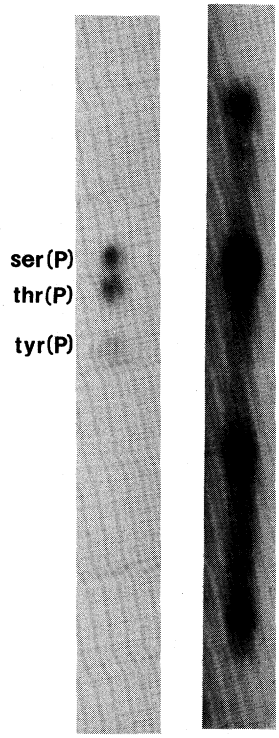

FIG. 5. Thin-layer electrophoretic separation of phosphorylated amino acids. Thin-layer electrophoresis at pH 3.5 was used to separate phosphoserine (Ser(P)), phosphothreonine (Thr(P)), and phosphotyrosine (Tyr(P)), for analysis of an acid hydrolysate of phosphorylated κ-casein (as described under Experimental Procedures). On the left are shown standard amino acids, visualized by reaction with ninhydrin. On the right is shown an autoradiogram (12 h exposure) of the track where the hydrolysate was run. The lowest spot of radioactivity corresponded to the origin. The radioactivity with highest mobility corresponded to inorganic phosphate. The radioactivity of low mobility (between the origin and phosphotyrosine) is unidentified but could represent an incomplete hydrolysis product.

of phosphorylase (40) or serine-7 of glycogen synthase (21, 41), two serines phosphorylated efficiently by phosphorylase kinase. It will therefore be of interest to identify the site (or sites) in κ-casein phosphorylated by phosphorylase kinase, for comparison with known sites of action.

As a substrate of phosphorylase kinase, κ-casein displayed several properties in common with glycogen synthase and phosphorylase. Specifically, the phosphorylation rate was stimulated by Ca^{2+} and calmodulin, was higher at pH 8.2 than at pH 6.8, and was increased by partial tryptic proteolysis. In comparing phosphorylase and glycogen synthase as substrates (18), we interpreted similar parallel behavior as evidence that a single catalytic center of phosphorylase kinase was involved in phosphorylating both substrates. From the results described here, the same logic would lead one to postulate that κ-casein is another substrate for this same catalytic center. Which subunit of phosphorylase kinase contains the active site is still a matter of discussion: evidence has been presented implicating both the γ (42) and the β (43) subunits. Other evidence has suggested that more than one active site is present in the molecule (see Ref. (44)). Whatever the outcome of this controversy, it is clear that several important mechanisms for phosphorylase kinase activation operate whether phosphorylase, glycogen synthase, or κ-casein is the substrate.

An important implication of this study is that caution is necessary when whole casein is used to screen for substrate specificities of protein kinases. This point has been made previously (26), and is very clearly reaffirmed by the present report. Whole casein, as well as histones, phosvitin, and protamine, find frequent use as readily available protein kinase substrates. Here, we described a preferential phosphorylation of κ-casein by phosphorylase kinase. Kemp *et al.* (45) demonstrated that cyclic AMP-dependent protein kinase phosphorylates specifically β-casein B and Bingham *et al.* (26) showed that this component accounts for most of the phosphorylation, by the same kinase, of whole casein. Therefore, two protein kinases, undeniably of quite different character, could be classified as "casein kinases" on the basis of screening only with whole casein. Besides these protein kinases, other casein kinases have been described with quite different specificities for individual casein components (46–51). A related example is a glycogen synthase kinase, labeled by us $PC_{0.7}$ (22), that preferentially phosphorylates both the A and B variants of β-casein (DePaoli-Roach, Bingham, and Roach, unpublished). The number of enzymes known to undergo covalent phosphorylation is increasing (2) and there is growing evidence for a multiplicity of protein kinases in the cell. Since many of the kinases await definitive characterization, substrate specificity will be an important criterion for characterizing these enzymes. For this reason, the use of heterogeneous substrates such as whole casein must be made with care. Why, as a family, caseins are substrates for such a range of protein kinases is in itself an interesting but somewhat separate question.

In summary, phosphorylase kinase can phosphorylate κ-casein at a rate that is modified by several of the known controls of phosphorylase kinase activity. Further study of this protein as a model substrate may provide a useful probe of the specificity of phosphorylase kinase.

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